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14. ABSTRACT The Polycomb Group (PcG) of gene silencers are chromatin associated multi-protein complexes that maintain cell identity by regulating the expression of genomic programming genes. In stem cells, PcG complexes occupy hundreds of genomic sites repressing genes required for differentiation. We aim to investigate the role of a disulfide bond that forms within a member of the PcG called Ph. We predict that the formation of the disulfide bond would have detrimental consequences to the ability of Ph to repress transcription which would hinder the ability of the stem cell to maintain their pluripotent state. Our proposed studies will provide insights into the molecular events that underlie stem cell function by shedding light on a potential target of reactive oxygen species. Greater knowledge of these processes will undoubtedly be helpful not only in expanding our understanding of how the PcG functions but also in the design of therapeutics targeted specifically to cancer stem cells.				
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INTRODUCTION

Polycomb Group (PcG) proteins maintain cell identity, including those of cancer stem cells, by regulating the expression of genomic programming genes. In stem cells, multi-protein PcG complexes bind to and repress hundreds of genes whose expression promote differentiation and if uncontrolled, lead to cancer. Understanding the role of the PcG in cancer and fulfilling the promise that stem cells hold for regeneration therapy will require understanding the molecular mechanism of the PcG. The goal of this proposal was to test whether a PcG protein called **Polyhomeotic (Ph)** is a target of reactive oxygen species and if oxidized, would hinder its ability to repress transcription. Our preliminary data does indeed suggest that Ph function can be affected by a cysteine residue that undergoes oxidation.

Ph contains a C-terminal Sterile Alpha Motif (**SAM**) domain. SAM domains are found in over 3000 proteins and are involved in a diversified array of binding reactions¹. Like many other SAM domains, Ph SAM is able to self-associate into polymeric architecture (Fig. 1)². This structure was solved at pH 4.0 which is a very low pH condition and likely to result in the protonation of any Cys residues that are present. Not surprisingly, the Cys residue within Ph SAM was observed to be kept in the reduced state and found buried in the core of the protein. In a second structure solved at a more neutral pH condition, the same Cys residue is now oxidized and forms a disulfide bond with the same Cys residue of another Ph SAM domain³.

While certainly possible that this disulfide bond is an artifact of crystallization, there are reasons to believe that the formation of this disulfide bond can actually play a role in Ph function. First, there is a high propensity for the disulfide to form. The Cys residue must undergo an energetically unfavorable conformational change from being buried in the core of the structure to being exposed in order to form the disulfide bond. Despite this energy barrier, the disulfide still forms even in the presence of 5 mM DTT. Second, there is extensive buried surface area between the two cross-linked Ph SAM molecules (1090 Å²) which is larger than many well-established protein-protein interactions suggesting this interaction is not the result of chance. Third, the Cys residue that undergoes the oxidation is conserved in all Ph orthologs. Fourth, a possible functional role is suggested by the structure of the disulfide linked Ph SAM dimer. An overlay of the reduced Ph SAM polymer structure on either of the two disulfide linked Ph SAM structures reveals a steric clash that would preclude Ph SAM polymerization (Fig. 2). This would be a novel mode of regulating SAM domain polymerization. Finally, despite the reducing environment, disulfide bond redox triggers do occur in cells⁴.

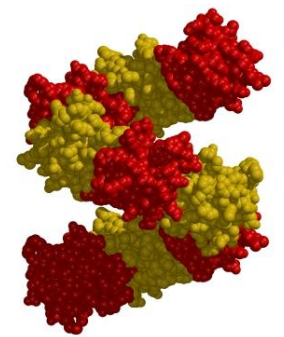


Fig. 1. Ph SAM polymer. Nine Ph SAM units arranged in a head-to-tail polymer arrangement are shown. Each SAM unit is shown in alternating colors.

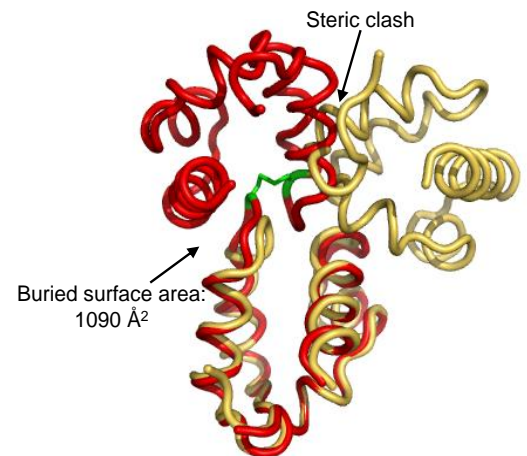


Fig. 2 Structural overlay analysis. The disulfide linked Ph SAM dimer is shown in red with the cross-linked Cys residues in green; two links of the fully reduced Ph SAM polymer structure are shown in yellow. An oxidized disulfide linked Ph SAM domain (top, red Ph SAM) is positioned such that it overlaps the binding surface for an additional reduced Ph SAM (top, yellow) in the polymer structure. Therefore, Ph SAM disulfide bond formation would preclude Ph SAM polymerization. The steric clash between the two mutually exclusive

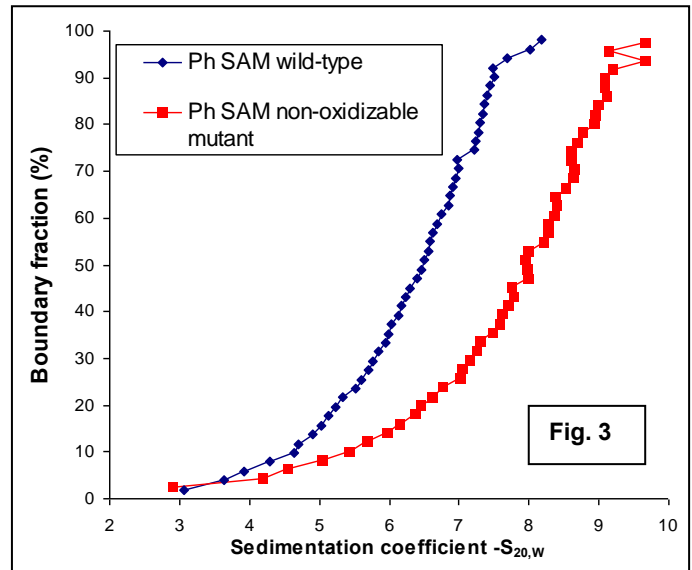
BODY

We proposed to study the potential redox regulation of the Polycomb Group (PcG) protein called Polyhomeotic (Ph). We hypothesized that the disulfide bond formed between two Ph molecules via their SAM domains plays an important role in inhibiting the repression activity of Ph which in turn would have significant consequences in stem cell maintenance including those of cancer stem cells. The following were our proposed tasks/aims:

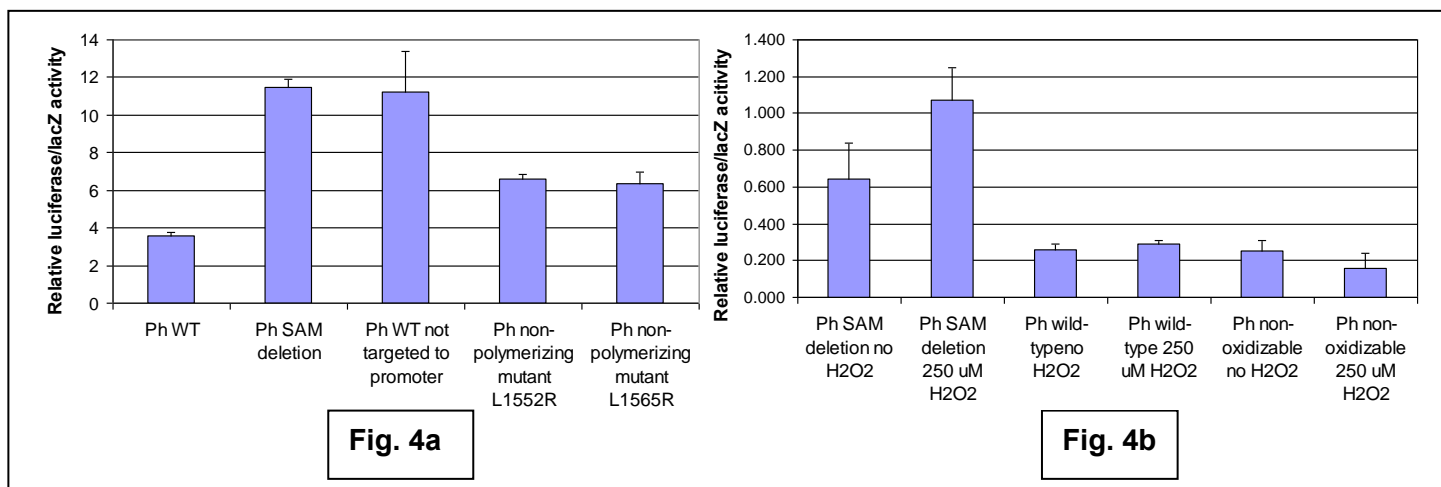
1. Show that the Ph SAM disulfide bond inhibits polymerization.
2. Show Ph SAM disulfide bond formation hinders the ability of Ph to repress transcription.
3. Show Ph SAM disulfide formation is relevant *in vivo*. This particular task will be in collaboration with Dr. Donald McEwen.

We have made significant advances in tasks 1 and 2 and have begun our collaboration Dr. McEwen for task 3.

We have completed task 1 and shown that formation of the disulfide bond between two Ph SAM domains limits its polymerization. We first mutated the Cys residue within Ph SAM to a Leu and Val. We chose these two hydrophobic residues because we wished to favor the reduced Cys conformation where the Cys is buried in the core of the protein. Both the Leu and Val Ph SAM mutants express solubly and in comparable amounts as the wild-type suggesting a minimal disruption to the overall fold of the protein. We further confirmed that the C to L mutation maintains the proper three dimensional fold of the SAM domain by performing binding experiments with the C to L mutant and assuring its ability to mediate the polymerization interactions (data not shown). We then proceeded to assess how the non-oxidizable Ph SAM domain mutant polymerizes compared to wild-type. According to our hypothesis, we predict that Ph SAM disulfide formation hinders Ph SAM polymerization. We assessed the degree of polymerization using analytical ultracentrifugation. The van Holde - Weischet combined distribution plot of Ph SAM wild-type versus the C to L mutant shows a higher distribution of S-values for the C to L, non-oxidizable mutant (Fig. 3) indicating a higher molecular weight species than the wild-type reflecting a higher degree of polymerization.



For task 2, we utilized a transcription assay to show that formation of the Ph SAM disulfide bond hinders the ability of Ph to repress transcription. Our first objective was to demonstrate that Ph SAM is required for Ph mediated repression. Ph SAM is required for repression as deleting it hinders the ability of Ph to repress transcription (Fig. 4a). Structure guided SAM domain mutants which hinders polymerization were also deficient in their ability to repress transcription. These results, for the first time, show that Ph mediated repression is dependent on the ability of the SAM domain to polymerize. We next sought to show that Ph SAM disulfide formation hinders repression. Our initial experiments showed very promising results consistent with our hypothesis. Wild-type Ph represses transcription to equal extents at 0 and 250 μM H_2O_2 (Fig. 4b). Strikingly, Ph with the C to L, non-oxidizable mutation in its SAM domain appears to be a better repressor in the presence of H_2O_2 . We are in the process of repeating these experiments and optimizing the assay protocol.



Finally, we have initiated our collaboration with Dr. Don McEwen to determine the effects of removing the Ph SAM disulfide bond has on *Drosophila*. We will introduce both wild-type and the non-oxidizable mutant Ph into a background *Drosophila* that lack Ph and test the stem cell function in the gut. We will feed paraquat to the flies to cause oxidative stress induced damage to the gut then compare the regenerative properties of the flies' gut cells from the different transgenic Ph flies. We estimate that this task will take at least six months to complete.

In summary, we have shown that removing the ability of Ph SAM to form a disulfide bond results in a greater ability to polymerize and that transcriptional repression is dependent on polymerization. Further, our preliminary data suggest that the disulfide bond formed within Ph SAM can lead to a decreased ability of Ph to repress transcription. Given the promising *in vitro* and tissue culture results, we anxiously await the results from our *in vivo* studies in *Drosophila*. We have requested a no-cost extension which would allow us to continue our studies on this subject.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration of a non-oxidizable Ph SAM domain does indeed polymerize better than wild-type
2. Establishment of a sensitive transcription assay for use with Ph
3. Demonstration of Ph SAM polymerization is required for transcriptional repression

REPORTABLE OUTCOMES

N/A

CONCLUSION

I hypothesize that the disulfide bond that can form between two Ph SAM domains can regulate the function of Ph. Our preliminary data supports this hypothesis. For example, Ph SAM mutants that cannot be oxidized show a greater tendency to polymerize. As we have shown that the ability of Ph to repress transcription is dependent on its SAM domain's ability to polymerize, we predict that the disulfide bond formation will lead to deficiencies in Ph ability to repress transcription. We hope to verify this shortly using our transcription assay and also test transgenic flies with non-oxidizable Ph proteins in their ability to withstand oxidative stress compared to wild-type.

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APPENDICES

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